IN THE U.S. PATENT & TRADEMARK OFFICE

Applicants:	Yukoh HIEI et al		
Serial No.:	10/089,696	Group:	1661
Filed:	July 24, 2002	Examiner:	Kubelik

For: Method for Promoting Efficiency of Gene Introduction into Plant Cells

DECLARATION UNDER 37 C.F.R. § 1.132

Honorable Commissioner of Patents and Trademarks

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Alexandria, Virginia 22313-1450

Sir:

I, Yukoh HIEI, a nation of Japan, residing at c/o Japan Tobacco Inc., Plant Breeding and Genetics Research Laboratory, 700, Higashibara, Toyoda-cho, Iwata-gun, Shizuoka 438, Japan, do hereby declare as follows:

I am a co-applicant of the invention as described and claimed in the specification of the above-identified application.

I am familiar with the Office Action dated January 8, 2009, in which claims 22 - 30 are rejected.

To show the patentability of the present invention, I carried out the experiments described below.

Materials and Methods

(1) *Agrobacterium* Strain and Plasmid

As the *Agrobacterium* and its vector, LBA4404(pSB134) (Hiei and Komari, 2006) was used. The T-DNA region of pSB134 has a hygromycin-resistant gene (*hpt*) regulated by maize ubiquitin promoter and a GUS gene regulated by the 35S

promoter of CaMV and having the first intron of the catalase gene of castor-oil plant.

(2) Sample Varieties and Tissue

As the sample variety, Yukihikari, which is the variety of Japonica rice, was used. As the sample tissue, immature embryo was used. The preparation method of the tissue is the same as that described in the specification of the present patent application.

(3) Centrifugation Treatment

Rice immature embryo was placed in a 1.5 ml centrifugal tube containing 1 ml of sterilized water. The tube was subjected to centrifugation treatment for 10 minutes or 4 hours at 20,000 xg at 25 degrees. In each experimental plot, 30 immature embryos were used. After the centrifugation, the immature embryos were infected with *Agrobacterium*.

(4) Infection of *Agrobacterium* and Co-culturing

The method of infection of the immature embryos with *Agrobacterium*, the method of co-culturing and the method of GUS assay of the immature embryos after the co-culturing were the same as described in specification of the present patent application. The co-culturing was carried out for 4 days. In the present test, the GUS expression levels in the immature embryos were expressed in values as GUS Activity Index as follows: Each of the immature embryos was then visually examined for the percentage of the sum of the blue areas to the total surface area of the scutellum. A score was given according to the percentage; score 0.0 was given for 0%, score 0.5 for between 0% and 1%, score 5.5 for between 1% and 10%, score 17.5 for between 10% and 25%, score 37.5 for between 25% and 50%, score 62.5 for between 50% and 75%, and score 87.5 for 75% and 100%. The average of the scores from 15 immature embryos in an experimental plot was recorded as the GUS Activity Index. The remained 15 immature embryos co-cultured were subjected to microscopic observation and transferred to non-selective medium nN6C (N6 salts and

vitamins (Chu, 1978), 0.5 g /l vitamin assay casamino acids, 0.5 g /l L-proline, 0.3 g /l L-glutamine, 20 g /l sucrose, 55 g /l D-sorbitol, 1.0 mg /l 2,4-D, 0.5 mg /l NAA, 0.1 mg /l BA, 250 mg/l cefotaxime, 100 mg/l carbenicillin 5.0 g /l Gelrite, pH5.8) and cultured at 32 degrees under the light condition for 3 days. After the culturing, the growth of the immature embryos was observed.

Results and Discussion

A tendency was observed that the growth of the hypocotyl is inhibited and the scutellum is grown during the co-culturing in the immature embryos subjected to centrifugation. The state of the immature embryos after co-culturing is shown in Figure1- (B) and (C). The immature embryos grew up to several times in comparison with immature embryos (Figure1-(A)) immediately after the isolation. The growth rate of the immature embryos was not observed in much difference between 10 minutes and 4 hours centrifugation (Figure1- (B) and (C)). The state of GUS expression in the immature embryos after the co-culturing is shown in Figure 2. As for the percentage of the area in the scutellum, which showed GUS expression, pretreatment of centrifugation for 10 minutes was somewhat higher than that of 4 hours (Figures 2 and Table 1). Thus, the centrifugation treatment at 20,000 xg has an effect to considerably increase the gene transfer efficiency even if it is performed for very long duration such as 4 hours. After the co-culture, the immature embryos subjected to further culturing for 3 days on culture media. Apparent differences of the growth of immature embryos were not observed between pretreatment for 10 minutes and 4 hours with centrifugation (data not shown).

Cited Reference

Chu C-C (1978) The N6 medium and its application to anther culture of cereal crops. In: Proc. Symp. Plant Tissue Culture, (pp. 43--50). Science Press, Peking

Hiei Y, Komari T (2006) Improved protocols for transformation of indica rice mediated by *Agrobacterium tumefaciens*. Plant Cell, Tissue Organ Culture 85, 271-283

Table 1. Transient GUS activity in immature embryos after co-cultivation with *A. tumefaciens* LB4404(pSB134). The immature embryos were pretreated with centrifugation.

Pretreatment with centrifugation		GUS Activity Index
		Variety
Centrifugal acceleration (xg)	Time	Yukihikari;
20,000	10 min	46.5
20,000	4 hours	30.7

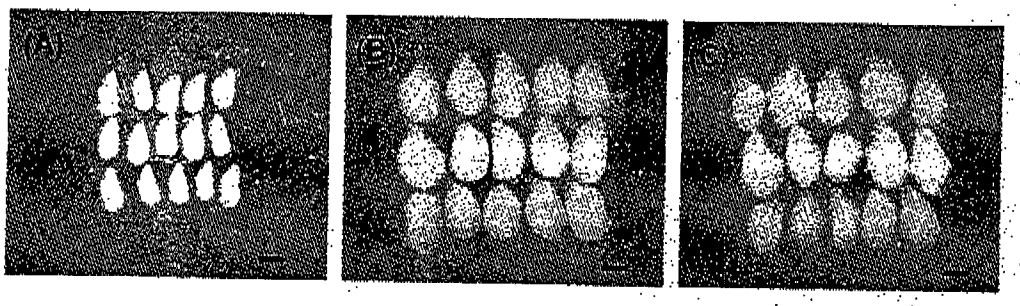
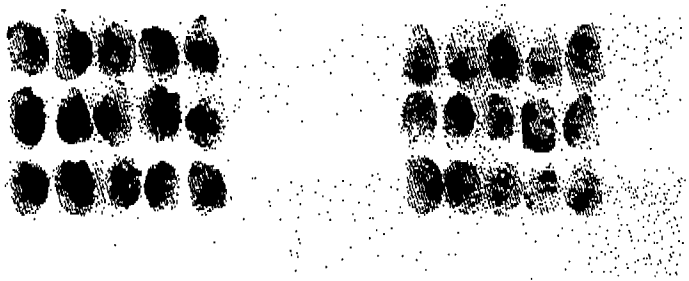


Figure 1. Immature embryos of Yukihikari. (A) immature embryos immediately isolated from immature seeds. (B) and (C) immature embryos after co-cultivation with *A. tumefaciens* LB4404(pSB134) for 4 days. The immature embryos were pretreated with centrifugation at 20,000 xg for 10 minutes (B) and 4 hours (C). Scale bar, 1 mm.



20,000 xg for 10 minutes 20,000 xg for 4 hours

Figure 2. Histochemical GUS expression in immature embryos of Yukihikari after co-cultivation with *A. tumefaciens* LB4404(pSB134) for 4 days. The immature embryos were pretreated with centrifugation.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

This 5 day of June, 2009

Yukoh HIEI